TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor

Development 115, 1111-1119 (1992)
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TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit ligand) is a survival factor

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Summary

We have used a probe derived from TRP-2/DT to detect migratory melanoblasts shortly after they emerge from the neural crest, as early as 10 days post coitum (dpc). TRP-2/DT expression is otherwise restricted to the presumptive pigmented retinal epithelium, the developing telencephalon and the endolymphatic duct. The pattern of *steel* and c-*kit* hybridisation in the developing brain differed from that of TRP-2. TRP-1 and tyrosinase probes also detected melanoblasts but were both expressed later in development than TRP-2. We used the TRP-2/DT probe to investigate the way that the *Steel-dickie* (Sl^d) mutation interferes with melanocyte development, and found that the membrane-bound steel growth factor which is missing in Sl^d/Sl^d mutants is necessary for the survival of melanoblasts but not for their early migration and initial differentiation.

Key words: Steel factor, stem cell factor, mast cell growth factor, *Steel-dickie* mutant, melanocytes, neural crest cell migration, TRP-2/DT, TRP-1, tyrosinase, c-kit ligand, mouse development.

Introduction

Melanocytes follow an interesting and diverse developmental pathway. Most melanocytes originate in the neural crest and migrate from there to populate specific regions of the developing mouse embryo, including the skin and hair follicles, the inner ear and the Harderian gland and choroid of the eye. Melanocytes that form the pigmented retinal epithelium (PRE) of the eye, in contrast, develop from locally derived neurectoderm.

No marker specific for early mammalian melanoblasts, the precursors of melanocytes, has been described. Such a marker would be invaluable for answering questions about the early stages of melanoblast migration in normal and abnormal embryos. What, for example, are the temporal and spatial relationships between migration, proliferation and differentiation of melanoblasts from their neural crest cell progenitors? Do a small number of cells migrate before they proliferate and differentiate, or is there a wave of proliferation earlier, within the neural crest, of cells destined to become melanocytes? Coat patterns of chimaeras suggest that large regions of the coat are pigmented by a single precursor, but does melanoblast proliferation occur before or after migration to the skin? Do melanocytes destined for different parts of the body follow the same developmental sequence?

Mutations at a number of loci give rise to white spotting of the coat, in which no melanocytes can be recognised in the white areas. It is not known whether the spotting is due to anomalous melanoblast migration, proliferation, survival or differentiation. Two particularly well-studied spotting genes, *W* and *Steel*, have been shown recently to encode a tyrosine kinase receptor, c-kit, and its ligand respectively (Chabot et al., 1988; Geissler et al., 1988; Copeland et al., 1990; Zsebo et al., 1990; Huang et al., 1990). This identification has not, however, allowed definition of the precise mechanism by which melanocytes fail to develop.

The mutation *Steel-dickie* (Sl^d) is a small deletion of the *Steel* gene which results in the encoded protein lacking its transmembrane domain (Flanagan et al., 1991; Brannan et al., 1991). The resulting protein does have some biological activity, but the lack of its transmembrane domain may interfere with secretion of the soluble form of the protein or alternatively the membrane-bound form may have a specific function in development resulting in the observed phenotypic effects (Brannan et al., 1991; Flanagan et al., 1991; Williams et al., 1990; Zsebo et al., 1990). Homozygous Sl^d/Sl^d mice have no coat pigmentation and presumably lack all neural crest-derived melanocytes. The postnatal stria vascularis of the cochlea is similarly completely lacking in identifiable melanocytes (Grainger, Barkway and Steel, unpublished data).

In this report, we characterise a new probe, derived from the tyrosinase-related protein-2 (TRP-2) gene. We have recently shown that TRP-2 is the melanogenic enzyme DOPAchrome tautomerase (DT), which converts DOPAchrome to 5, 6-dihydroxyindole carboxylic acid (Tsukamoto et al., 1992). In the absence of the enzyme,

DOPAchrome spontaneously converts to 5, 6-dihydroxyindole. TRP-2/DT is encoded at the *Slaty* locus on mouse chromosome 14 (Jackson et al., 1992). The only known mutant allele at the locus has much reduced but still significant DT activity, which results in the mice having dark grey/brown rather than black eumelanin.

Here we show that the TRP-2/DT probe detects migratory melanocytes and their precursors as early as 10 days of gestation and is also expressed in neurectoderm destined to become the PRE of the eye. We have focussed particular attention on the developing inner ear, as it represents a specific, well-defined and localised target for migratory melanocytes. In addition, it labels the telencephalon of the developing forebrain and the endolymphatic duct. In mice homozygous for the *Steel-dickie* mutation, melanoblasts were identified as TRP-2 labelled cells at 11 days of gestation, but there were fewer of them than normal and they were mostly restricted to a site just caudal to the otic vesicle, whereas in normal mice there were many labelled cells both caudal to and around the otic vesicle. From 12 days onwards, very few melanoblasts were seen in the mutants, and none were seen in postnatal specimens. We conclude that the *Steel-dickie* mutation does not prohibit early melanoblast differentiation, as the detected melanoblasts were expressing TRP-2/DT, and neither does it stop early migration. However, the mutation does affect melanoblast survival and targeting.

Materials and methods

Mice

We used ten Sl^d/Sl^d mutants aged 11 to 14 dpc and seven postnatal Sl^d/Sl^d mutants aged newborn to 4 days old. Littermate controls were either $Sl^d/+$ or +/+, and we used a total of 23 of these aged 11 to 14 dpc plus four between birth and 4 days old. In addition, a total of 31 CBA/Ca mice were used at daily intervals from 8.5 dpc to 3 days after birth. Sections from all of these specimens were hybridised with TRP-2 and the TRP-2 sense strand as a control. Of the CBA/Ca mice used, a subset of 21 from 10.5 dpc to 3 days after birth were also hybridised with TRP-1 and tyrosinase probes, and a second subset of six mice aged 9.5 to 13.5 dpc plus one 3-day-old mouse were used for the steel and c-kit hybridisations.

After processing, sections of the embryos were examined and the developmental stage determined (Theiler, 1989). Some of the younger mice (11.5 dpc and younger) were slightly retarded in development in relation to gestational age, and so we assessed the stage of development according to Theiler (1989) and describe them below as the age appropriate to their development rather than as their actual gestational age.

In situ hybridisation probes

^{35}S-labelled RNA probes were made as previously described, using T7 or T3 RNA polymerase to transcribe plasmid templates. The templates were as follows: tyrosinase probe was transcribed by T3 RNA polymerase from the plasmid pmctyr 101 following digestion with *Bam*HI (Ruppert et al., 1988, from G. Schutz). The TRP-1 probe was made from pTRP1.6, the 1.6kb *Hin*dIII fragment of pMT4 subcloned into pBS, digested with *Bam*HI and transcribed by T7 RNA polymerase (Shibahara et al., 1986). The TRP-2 probe was transcribed by T7 RNA polymerase from *Hin*dIII-digested p5A7, a subclone in pBS of the 1200 bp *Eco*RI cDNA insert of l-clone 5A (Jackson, 1988; Jackson et al., 1992). Control (sense strand) probe was also transcribed by T7 RNA polymerase from *Hin*dIII-digested p5A3, the same 1200 bp fragment cloned into pBS in the opposite orientation. The probe for c-kit was transcribed by T7 RNA polymerase from *Hin*dIII-digested pGEM-ckitUTR, the 1388 bp *Eco*RI-*Hin*dIII fragment from the 3′ untranslated region of mouse c-kit cDNA provided by P. Besmer through P. Koopman (Qui et al., 1988). The template for the steel probe was made by firstly PCR amplification of a 495 bp fragment from Buffalo rat liver cDNA using primers: TGGGAAAATAGTGGATGACC (bases 326-346) (Huang et al., 1990) and TATGAGCTCTTTCTGTTGCAGCATAC (complement of bases 616 to 597 plus a terminal *Sac*I site). The product was digested with *Nsi*I and *Sac*I and the resulting 467 bp fragment cloned into pBS. The clone was digested with *Hin*dIII and transcribed with T7 RNA polymerase.

In situ hybridisation

Pregnant mice were killed by cervical dislocation. The embryos were removed into cold phosphate-buffered saline (PBS) and the stage of development noted using features such as hindlimb and digit development (Rugh, 1990; Theiler, 1989). The embryos were fixed in 4% paraformaldehyde in PBS at pH 7.2 overnight, dehydrated and embedded in paraffin wax. Sections were cut at 8 μm thickness and mounted on TESPA (3-aminopropyltrimethoxysilane; Sigma)-coated slides, and the slides were then left at 56°C overnight to fix the sections to the glass. Sections were mounted in small groups to allow hybridisation of adjacent sections with different probes, and some sections were incubated with a control, sense strand. For most of the CBA embryos and all of the embryos from the Sl^d stock, serial sections were collected through the entire head region. Only a small number of the youngest embryos from the CBA stock were sectioned whole, so our observations of the trunk region are limited.

In situ hybridisation was performed by the method described by Wilkinson et al. (1987a), with high stringency washes as described by Wilkinson et al. (1987b). Briefly, the prehybridisation treatment included steps to fix the material further, to digest the sections partially with proteinase K to improve access of the probe to mRNA, and to block basic groups by acetylation to reduce non-specific binding of the probe. Sections were incubated with the appropriate ^{35}S-labelled RNA probe in hybridisation mixture at 55°C overnight. The slides were washed under stringent conditions and treated with RNAase to remove unhybridised, non-specifically bound probe, then dehydrated and dried. The slides were coated with Ilford K5 liquid emulsion for autoradiography, and exposed for 3-4 weeks. After development, the sections were counterstained with methyl green and mounted under a coverslip with DPX. The sections were examined by bright- and dark-field illumination.

Genotyping of Sl^d *embryos*

As homozygous Sl^d/Sl^d mice are sterile, we crossed animals heterozygous for the mutation, and took embryos at a number of developmental stages. The extraembryonic membranes and surplus embryonic tissues were used to prepare DNA for genotyping, the caudal part of the embryo was processed for identification of germ cells, and the head region of the embryos was processed for in situ hybridisation. Initially, all the embryos were hybridised and examined without knowledge of their genotype. Postnatal mutant mice were distinguished from control littermates by their pale appearance due to anaemia and lack of pigmentation.

We developed a PCR-based assay for identification of embryos homozygous for the Sl^d mutation. DNA was prepared from the limb buds of individual embryos by proteinase K digestion in the presence of 0.5% SDS followed by phenol and chloroform extractions and ethanol precipitation. PCR was carried out with primers CCATGGCATTGCCGGCTCTC (bases 665 to 684) (Huang et al., 1990) and CTGCCCTTGTAAGACTTGACTG (complement of bases 757 to 736). The reaction amplifies a DNA segment across an intron, resulting in a product of about 700 bp from wild-type DNA (C Brannan, personal communication). The latter primer is within the exon deleted in the Sl^d mutation, and therefore no amplification product is produced from the mutant chromosome. To control for PCR efficiency, a second primer pair was included in each reaction. These primers, TCCGAATTCAAAGGGGTG-GATGACC and GACACATAGTAATGCATCC, amplify a 347 bp fragment from the tyrosinase gene (Jackson and Bennett, 1990). The reactions were carried out using Taq polymerase from Promega in the buffer as supplied, plus each primer at 3 μg/ml and dNTPs at 0.2 mM. The reaction was incubated at 94°C for 2 minutes, followed by 30 cycles through 92°C for 1 minute, 53°C for 1 minute and 72°C for 1 minute. The reaction products were analysed on 4% NuSieve agarose gels. Fig. 1 shows an example of the analysis. Homozygous mutant embryos amplify only the tyrosinase fragment, whilst heterozygotes and wild-type embryos have both. We do not consider it possible to differentiate between the latter two genotypes by this assay.

The method was validated, and some embryos were also genotyped, by Southern blot hybridisation of *Eco*RI-digested DNA, including DNA from adult mice of known genotype, with the 1.4 kb KL cDNA from the *Sl* locus kindly provided by P. Besmer (Huang et al., 1990). The Sl^d allele is missing the largest hybridising fragment of 11-13 kb. (Zsebo et al., 1990; Copeland et al., 1990).

The genotype of mutant embryos was confirmed by examination of alkaline-phosphatase-stained sections of the genital ridge/gonad region. Sl^d/Sl^d mutants had very few or no alkaline-phosphatase-positive germ cells (McCoshen and McCallion, 1975) while littermate controls did have recognisable germ cells.

Fig. 1. 4% NuSieve gel electrophoresis of the products of PCR amplifying simultaneously with tyrosinase (lower fragment) and steel primers. M, PhiX174/*Hae*III size markers; 1, wild-type DNA; 2, heterozygous ($Sl^d/+$) DNA; 3, homozygous (Sl^d/Sl^d) DNA; 4-8, DNA from the forelimbs of five 11 dpc embryos segregating Sl^d and wild-type. Homozygous mutant embryos are in lanes 5-7. The band near the origin of lane 3 we presume is non-specific amplification product or is unamplified genomic DNA.

Results

Expression of melanocyte-specific genes

We used three probes derived from melanocyte cDNA libraries: tyrosinase, tyrosinase-related protein-1 (TRP-1) and TRP-2. The three encoded proteins are clearly related, having about 40% amino-acid identity between them. Given the known properties of tyrosinase, we assume that all three proteins are membrane-bound copper-containing enzymes, localised in melanocytes to the inner face of the melanosome (Hearing and Jimenez, 1989). Tyrosinase is the product of the *Albino* locus, in which mutations result in reduced or absent pigmentation. Mutations of TRP-1, (which is the product of the *Brown* locus) and TRP-2 (the product of the *Slaty* locus) affect the quality of pigment made (Jackson, 1988; Jackson et al., 1992).

When we examined the expression of these genes in late stage embryos and in newborn mice, we observed an overlapping distribution of hybridisation to melanocytes, identifiable by the presence of melanin (Fig. 2C,D,F). All melanin-containing cells showed strong hybridisation with TRP-2. TRP-1 and tyrosinase labelling was restricted to melanocytes, but some melanin-containing cells in the skin and hair follicles showed little labelling with TRP-1 or tyrosinase probes, possibly indicating cyclic activity of these genes in skin melanocytes.

At earlier times of development, melanocyte precursors (melanoblasts) show no visible melanin and cannot normally be distinguished from surrounding mesenchymal cells. However, the TRP-2 probe allows us to identify these precursors from as early as 10 days post coitum (dpc). We propose that these individual TRP-2-labelled cells are melanoblasts for a number of reasons. Firstly, at later stages of development, all melanin-containing cells showed TRP-2 activity. Secondly, TRP-2 activity was detected in individual cells as expected, rather than occuring in clumps or bands of mesenchymal cells. Thirdly, appropriate numbers of cells were detected for them to be melanoblasts (for example, we would not have expected the majority of mesenchymal cells in the head to be melanoblasts). Fourthly, we detected TRP-2 labelled cells at locations where we would expect to find melanocytes such as the eye, hair follicles and inner ear, and at early stages labelled cells were found in locations consistent with their presumed route of migration, such as in the mesenchyme alongside the neural tube and around the eye and inner ear (Figs 2,3); equally, we did not detect any TRP-2 expression in other cell types known to be derived from the neural crest, such as ganglia or mesenchymal cells of the lower jaw, suggesting that TRP-2 is specific to the melanocyte lineage rather than a general neural crest marker. Fifthly, the labelled cells were detected at the time in development when we would have expected melanocyte precursors to be migrating, late in neural crest migration. Finally, no TRP-2 labelled cells were seen in postnatal Sl^d/Sl^d mutant heads, corresponding to a lack of detectable melanocytes in these mice using other techniques such as electron microscopy; in addition,

the only cell types known to be affected in *Steel* mutants are melanocytes, germ cells and haematopoeitic cells, so the deficiency of TRP-2 labelled cells in the head of Sl^d/Sl^d mutants at 12,13 and 14 dpc corresponds well to the known effects of the gene if the labelled cells are melanoblasts as we propose. We cannot rule out the possibility that the TRP-2/DT probe labels individual cells that do not ultimately differentiate into melanocytes, but we suggest that the weight of the evidence supports our proposal that it is most likely a specific marker for melanoblasts. Two other distinct regions of hybridisation of TRP-2 are described below, but these are regions of uniform labelling of bands of neurectoderm and are readily distinguished from the individual labelled cells migrating through the mesenchyme.

TRP-1 and tyrosinase probes also label melanocytes before pigment is visible, but after TRP-2 labelling is apparent (Figs 2,3). The earliest stages of development at which labelling occurs is given in Table 1. In migratory melanocytes, TRP-2 labelling is seen from 10 dpc, TRP-1 and tyrosinase labelling from 14.5 dpc, and pigment is visible from 16.5 dpc. In the presumptive PRE, TRP-2 labelling is seen even earlier, at 9.5 dpc, with TRP-1, tyrosinase and visible pigment appearing later (see Table 1).

Developmental pattern of TRP-2 expression

TRP-2 expression is first detected in the optic vesicle at around 9.5 dpc. The optic vesicle develops from a protrusion of the diencephalon of the forebrain, which flattens at the epidermal surface and invaginates to form the bilayered optic cup (Fig. 2A,B). The mesial layer subsequently differentiates into the PRE. We find TRP-2 expression at an early stage of this process, in a restricted part of the optic vesicle, prior to its invagination (Fig. 2A). Later the expression is clearly localised to the presumptive PRE (Fig. 2B) but still well before the appearance of visible pigment. Labelling is also found in cells that will develop into the iris (Fig. 5F). This supports our suggestion that TRP-2 labels melanoblasts at a stage when they are otherwise undifferentiated.

Large numbers of individual migrating melanoblasts are detected by TRP-2 in the mesenchyme alongside the hindbrain from 10 dpc. They are identified from about half of the distance from the crest to the otocyst (the early inner ear), but not as they leave the neural crest. They become distributed around the developing otocyst and around the eye (Fig. 3). Later, from 12 dpc onwards, we see melanoblasts in the dermis and mesenchyme just below the skin. However, they are not seen in such large numbers as around the ear and eye, and their distribution in skin of the head and neck region (on which most of our observations were made) is uneven; there are many areas of skin devoid of identifiable melanoblasts at this stage. Melanoblasts in the skin are first seen anterior to the eye and in the developing pinna, then in dorsal skin, and from about 13.5 dpc many were detected in the dorsal tail area. In late gestation, melanocytes were often localised to hair follicles, but many were still seen in the dermis.

Table 1. *Time of earliest expression of melanocyte markers in the PRE, migratory melanoblasts and telencephalon*

	TRP-2	TRP-1	Tyrosinase	Pigment
PRE	9.5	11.5	13.5	11.5
Melanoblasts	10	14.5	14.5	16.5
Telencephalon	10.5	Never	Never	Never

The times are given in days post coitum and are nominal ages taken from the equivalent stages of development in Theiler (1989).

The distribution of melanoblasts around the inner ear is illustrated in Fig. 3. At 10 to 11 dpc numerous melanoblasts are seen widespread in the mesenchyme around, but in particular caudal to, the developing otocyst, around the anterior cardinal vein (Fig. 3A,B,C). With time the melanoblasts localise, presumably by migration, to specific sites within the inner ear. They appear to spread in a rostral direction to surround the otic vesicle, and are located mainly ventral and caudal to the vesicle by 11.5 dpc. They become progressively more localised (Fig. 3C,D,F,G), and eventually, in late prenatal stages, they are largely restricted to the stria vascularis of the cochlear duct (Fig. 3G) and to particular regions of the vestibular part of the inner ear (not shown).

Migratory melanoblasts are also found, but in smaller numbers, in the mesenchyme between the neural tube and the developing eye. They eventually become distributed behind the PRE where they form the choroid (although there are surprisingly few recognisable choroidal melanoblasts before birth), and scattered within the Harderian gland, an accessory gland of the eye (Fig. 2).

Unexpectedly, TRP-2 is also expressed in the developing forebrain (Figs 4C,E, 5A,). We find this surprising as these cells are not destined to become melanocytes. We find TRP-2 labelling the frontal part of the telencephalon from approximately 10.5 dpc. The labelling persists until 14 dpc, and the labelled region appears to shift to the medial part of the lobes of the telencephalon between 10.5 and 14 dpc. We do not know if the mRNA that we detect is translated into protein, or what the function of the protein might be in the brain.

TRP-2 is expressed in the epithelium of the developing endolymphatic duct in specimens between approximately 11 and 14 dpc, but in no other epithelial tissue in the ear. The distribution of label indicated that this expression was not derived from migratory melanoblasts but from epithelial cells (Fig. 5C).

Expression of c-kit and steel

Mice with mutations in the genes encoding the growth-factor receptor c-kit (*W* locus) and its ligand, steel, have a deficit of neural crest-derived melanocytes, although locally derived melanocytes in the PRE are capable of producing pigment. Other neural crest-derived cell types are not apparently affected by these mutations. We have asked if the c-kit/steel interaction is required for the initial differentiation of migratory melanoblasts from other neural crest cells, and perhaps also for the expression of TRP-2 in the telencephalon. One prediction of this proposition is that c-kit mRNA should be expressed in the same cells that express TRP-2. To test this we compared the localisation of c-kit, steel and TRP-2 mRNAs in adjacent sections by in situ hybridisation with appropriate probes. Other reports have

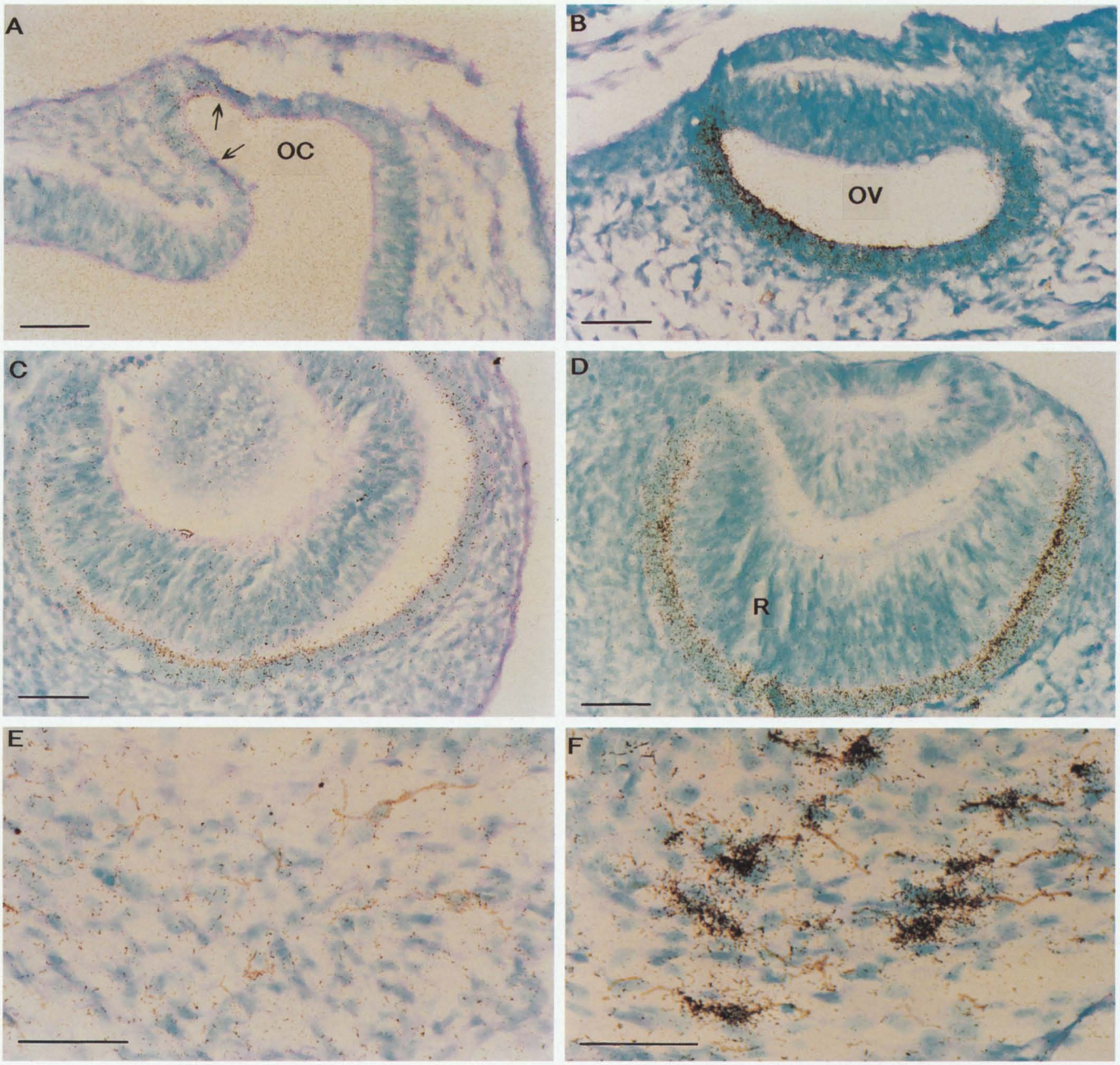

Fig. 2. In situ hybridisation of the developing eye and Harderian gland. OC, optic cup; OV, optic vesicle; R, retina. Scale bars, 50 μm. (A) Optic cup and optic stalk, CBA 9.5 dpc, TRP-2 hybridising to the presumptive PRE, shown by the black grains, between the arrows . This is the earliest sign of TRP-2 activity. (B) Optic vesicle, CBA 10 dpc, TRP-2 hybridising to PRE. (C) Eye, CBA 11.5 dpc, the earliest sign of TRP-1 transcription (black grains) and the first detectable visible pigment (brown colour). TRP-1 hybridises to the PRE throughout its thickness, while some pigment is seen at the inner edge of the PRE only. (D) Eye, CBA 11.5 dpc, TRP-2 hybridising to the entire width of the PRE. An isolated choroidal melanoblast appears at the bottom right. (E) Harderian gland, CBA 16.5 dpc, control hybridisation with the sense strand of TRP-2 showing the earliest detectable pigment (brown) in migratory melanoblasts. (F) Harderian gland, CBA 19.5 dpc, TRP-2 localising to migratory melanocytes identified by their pigmented dendrites.

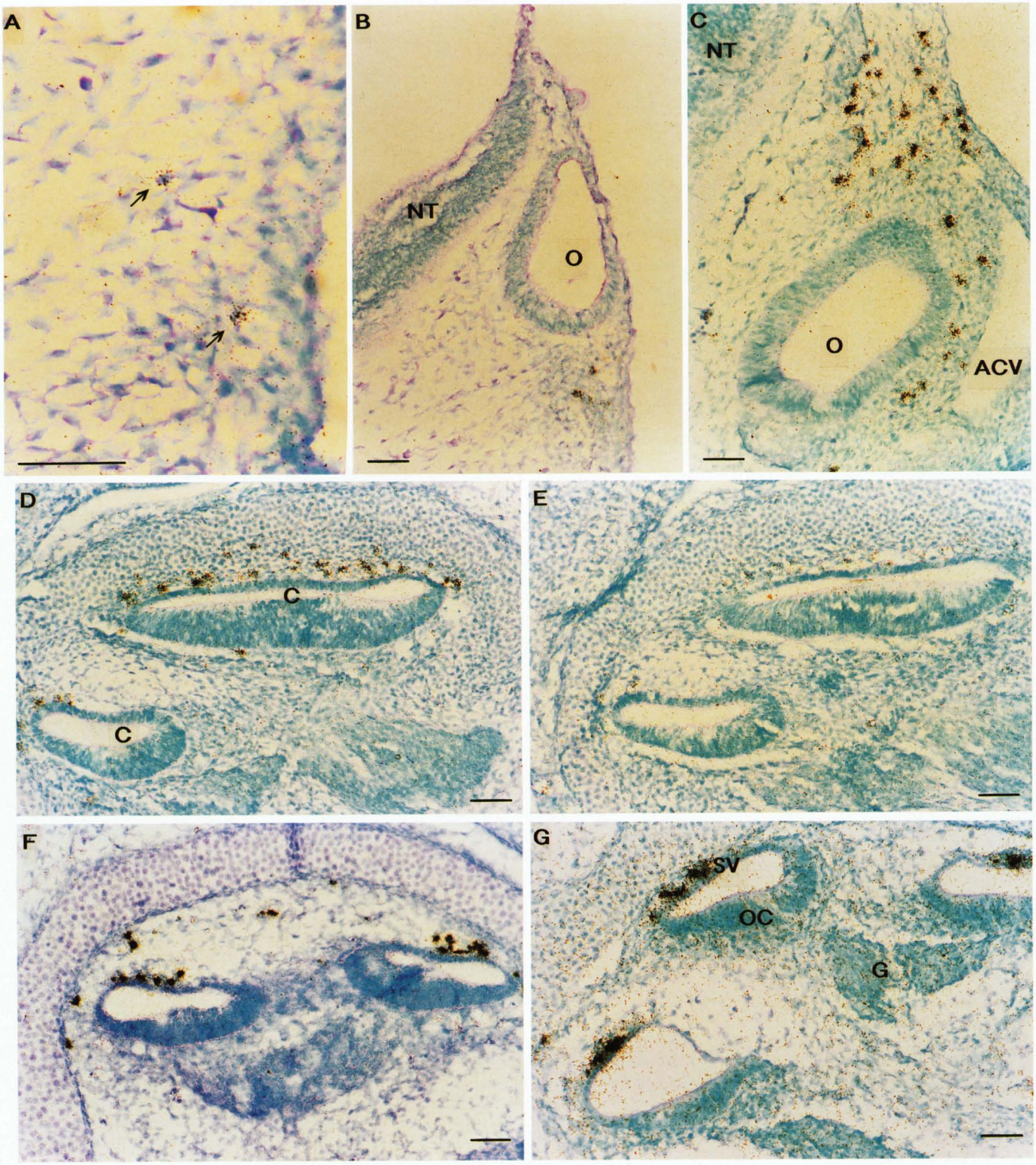

Fig. 3. In situ hybridisation of the developing inner ear and surrounding mesenchyme, showing the progressive localisation of melanoblasts to the stria vascularis of the cochlear duct. O, otocyst; NT, neural tube; AVC, anterior cardinal vein; C, cochlear duct; SV, stria vascularis; OC, organ of Corti; G, ganglion. Scale bars, 50 μm. (A) Mesenchyme caudal to otocyst, CBA 10 dpc, the earliest TRP-2 expression in migratory melanoblasts (arrows). (B) Otocyst and neural tube, CBA 10 dpc, TRP-2-labelled melanoblasts ventral to otocyst. (C) Otic vesicle, CBA 11.5 dpc, TRP-2-labelled melanoblasts distributed widely in the mesenchyme around the otic vesicle. (D) Cochlea, CBA 14.5 dpc, TRP-2-labelled melanoblasts are localised mainly to the mesenchyme above the cochlear duct. (E) Cochlea, CBA 14.5 dpc, tyrosinase hybridisation of a section near that in (D), showing the earliest activity detected in migratory melanoblasts. The distribution of labelled cells is similar to that for TRP-2. (F) Cochlea, CBA 15.5 dpc, TRP-2 hybridisation showing melanoblasts becoming more localised to the region of the cochlear duct that will develop into the stria vascularis. (G) Cochlea, CBA 16.5 dpc, TRP-2-labelled melanocytes becoming incorporated into the epithelium of the stria vascularis, particularly in the basal turn of the cochlea (bottom left) which is more advanced in development than the apical turns (top).

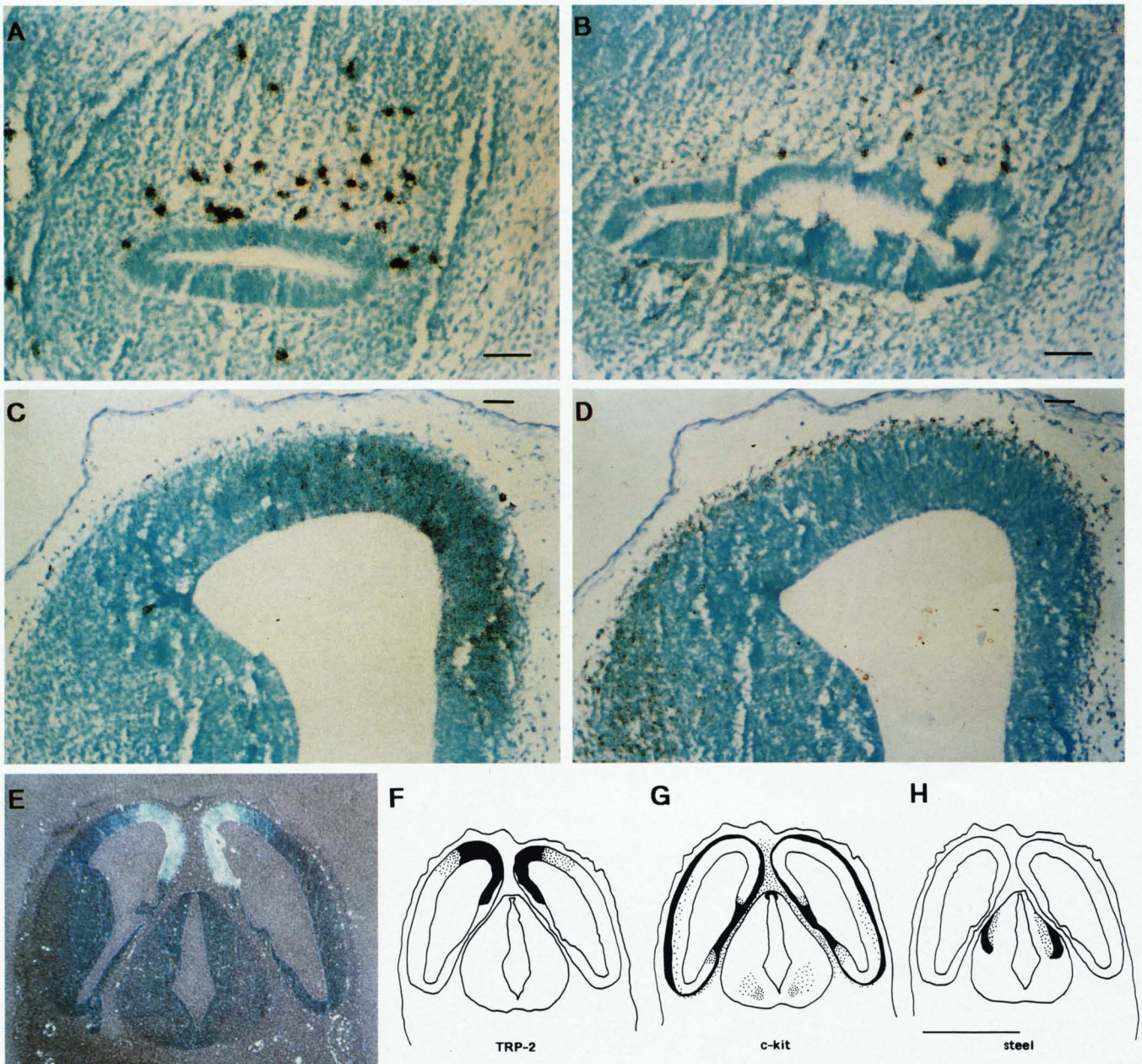

Fig. 4. (A-B) Cochlea, CBA 13.5 dpc, nearby sections of the cochlear duct labelled with (A) TRP-2/DT and (B) c-kit probes. Melanoblasts are detected as individual cells ventral to the cochlear duct (top of micrograph) with both probes, and c-kit additionally labels a band of mesenchyme on the opposite side of the duct, below the developing organ of Corti. Vertical streaks are section artefacts. Steel is strongly expressed in a band of mesenchyme ventral to the cochlear duct, where TRP-2 labelled cells are located (not shown). Scale bar, 50 μm. (C-D) Telencephalon, CBA 13.5 dpc, nearby sections labelled with (C) TRP-2/DT and (D) c-kit probes. (C) and (D) show the frontal part of the telencephalon, one side only, with the midline on the right, and illustrate the complementary pattern of labelling in this region, with c-kit labelling the outermost layer and TRP-2/DT the inner layer of the neuroepithelium. Scale bars, 50 μm. (E) Head, CBA 12.5 dpc, labelled with TRP-2/DT, to show the restriction of expression to the frontal and medial regions of the telencephalon. (F-H) Camera lucida drawings of adjacent sections of the brain in a 12.5 dpc CBA/Ca embryo. The two lobes of the telencephalon are to the left and right, and the diencephalon is in the centre. The distribution of strong hybridisation is shown by the solid black areas and moderate labelling is indicated by the dotted areas. (F) TRP-2 labelling. (G) c-kit labelling. Note labelling in the mesenchyme between the telencephalon and diencephalon as well as in the brain itself. (H) steel labelling. Label is restricted to the diencephalon. Scale bar, 1 mm.

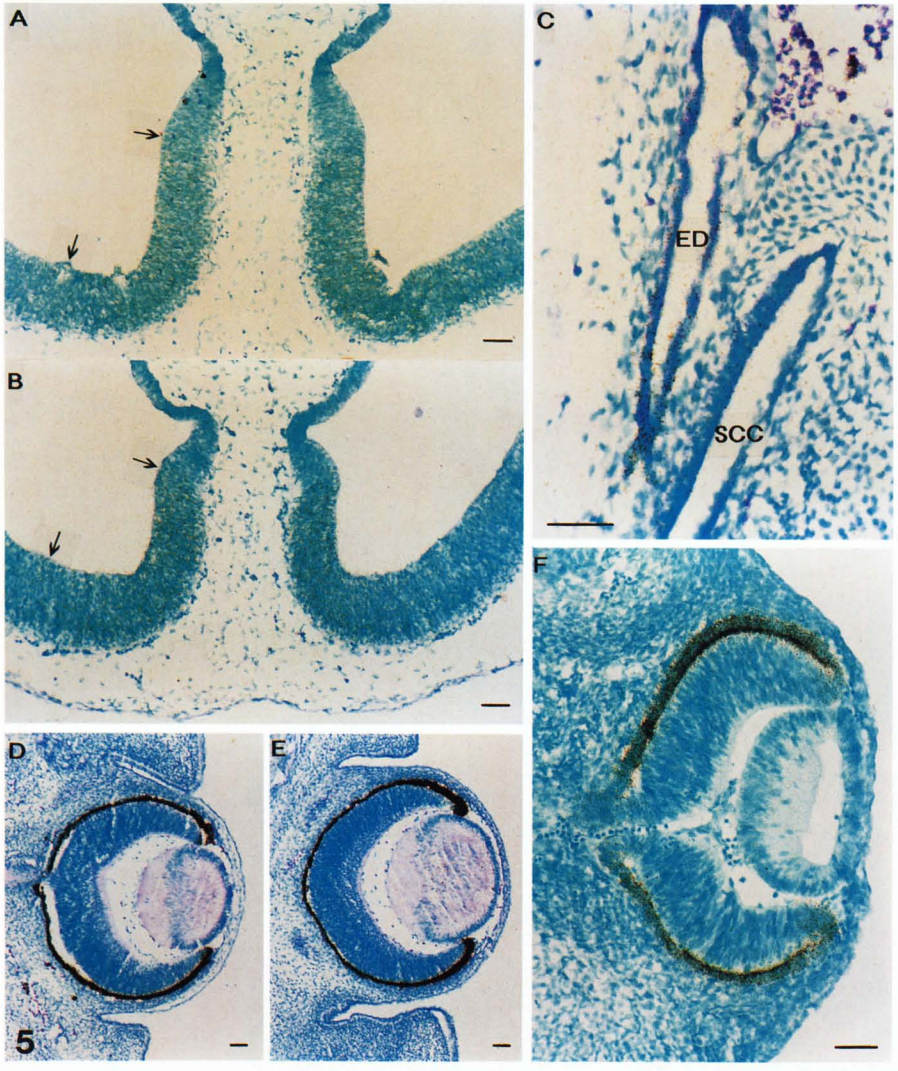

Fig. 5. Regions of TRP-2 expression in Sl^d/Sl^d mutants that appear normal. ED, endolymphatic duct; SCC, semi-circular canal. Scale bars, 50 μm. (A) Telencephalon, littermate control 12 dpc, TRP-2 hybridisation showing the normal restricted pattern of activity in the whole thickness of the neurectoderm between the arrows. (B) Telencephalon, Sl^d/Sl^d 12 dpc, TRP-2 hybridisation shows normal pattern. (C) Endolymphatic duct and semi-circular canal, Sl^d/Sl^d 14 dpc, TRP-2 expression is seen in the endolymphatic duct as in normal embryos. (D) Eye, littermate control 14 dpc, strong expression of TRP-2 in PRE. (E) Eye, Sl^d/Sl^d 14 dpc, TRP-2 expression in PRE is the same as normal, but there are no choroidal melanoblasts visible. (F) Eye, Sl^d/Sl^d 12 dpc, normal pattern of TRP-2 expression in the PRE, and also expression in the developing iris at the front edge of the retina.

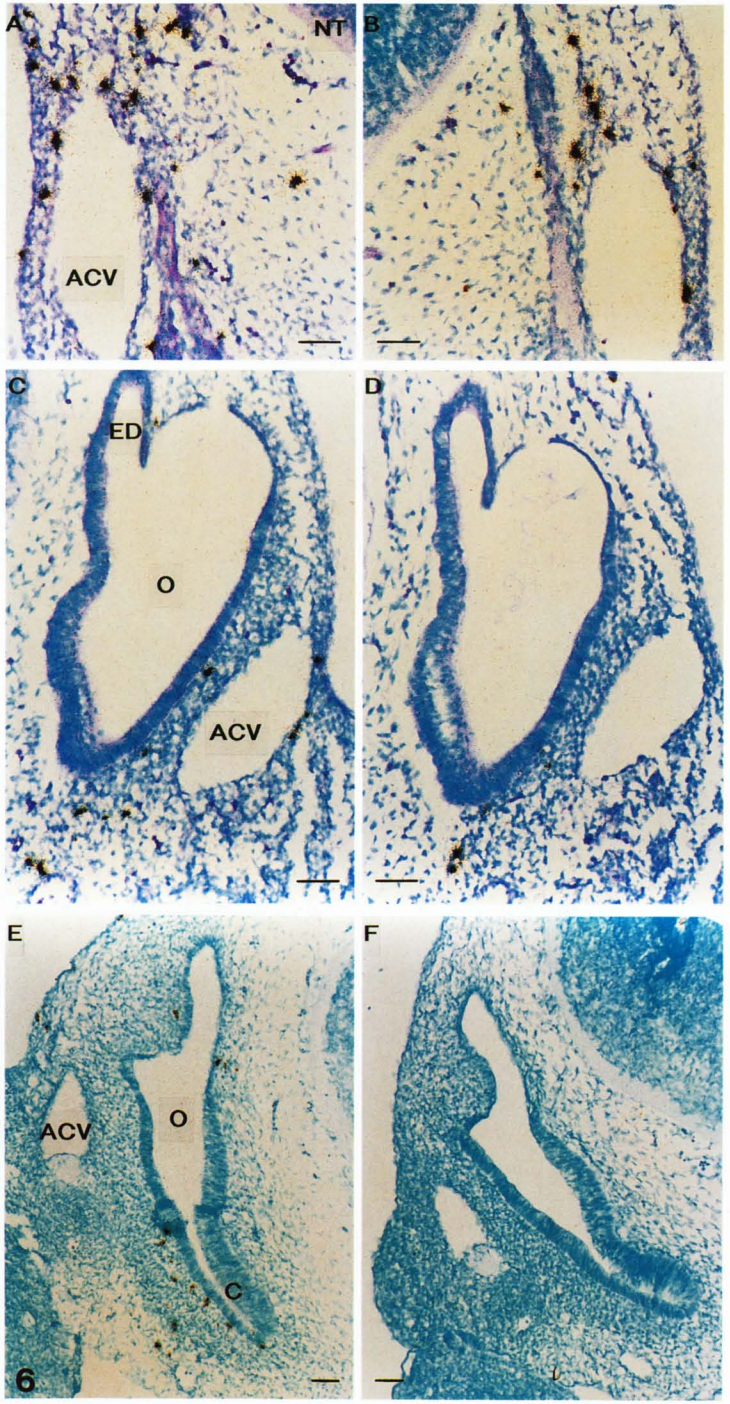

Fig. 6. Distribution of melanoblasts around the inner ear in Sl^d/Sl^d mutants. AVC, anterior cardinal vein; ED, endolymphatic duct; O, otic vesicle; C, cochlear duct. Scale bars, 50 μm. (A) Mesenchyme caudal to otic vesicle, littermate control, 11 dpc, TRP-2 hybridisation shows a number of melanoblasts clustered around anterior cardinal vein. (B) Mesenchyme caudal to otic vesicle, Sl^d/Sl^d 11 dpc, TRP-2 labels a similar number of melanoblasts at this site as in controls. (C) Otic vesicle, littermate control, 11 dpc, TRP-2-labelled melanoblasts begin to cluster around the developing cochlear duct. (D) Otic vesicle, Sl^d/Sl^d 11 dpc, fewer TRP-2-labelled cells are present at this location than in controls, and they rarely occur close to the cochlear duct. (E) Otic vesicle, littermate control, 12 dpc, TRP-2 hybridisation showing melanoblasts around developing ear, especially near the cochlea. (F) Otic vesicle, Sl^d/Sl^d 12 dpc, TRP-2 labels very few cells at this age, and none are near the inner ear.

given detailed accounts of the distribution of c-kit and steel transcripts in the developing mouse, so our intention was to focus only upon the relationship between the distributions.

At 9.5 dpc, before we see expression of TRP-2 in migratory melanoblasts, c-kit transcripts can be detected in mesenchymal cells in regions where subsequently TRP-2 expression is found. However, at later stages of development, c-kit is expressed in positions where no TRP-2 hybridisation is detected and in many more cells than can be accounted for by melanocyte precursors (eg Fig. 4B). For example, around the cochlear duct there are some individual c-kit labelled cells with a similar distribution and number to the TRP-2 labelled cells in adjacent sections, but there is also a broad band of c-kit labelling in the mesenchyme on the opposite side of the cochlear duct, below the developing sensory epithelium (Fig. 4B). These observations suggest that the pattern of c-kit expression is not responsible for selecting the melanocyte lineage, unless a restricted distribution of its ligand, steel, in concert directs melanocyte differentiation. This, however, is unlikely to be the case as hybridisation with steel probe reveals a widespread distribution of its transcript. Therefore, the distributions of steel and c-kit labelling suggest that the c-kit-steel interaction is not likely to be responsible for the initial differentiation or selection of melanoblasts from other neural crest cells.

Although steel is widely expressed, particularly strong expression was evident in the mesenchyme where TRP-2-expressing cells were found. At 11.5 dpc, steel is expressed in broad areas of mesenchyme corresponding generally with the migratory pathways of melanocytes. For example, at the level of the otic vesicle steel transcripts were found in the mesenchyme between the hindbrain and the surface ectoderm, particularly lateral and ventral to the otic vesicle around the site of the developing cochlea (data not shown). These were regions where, in adjacent sections hybridised with TRP-2 probe, many TRP-2-expressing cells were evident. In 12.5 and 13.5 dpc embryos, comparison of adjacent sections hybridized with steel, TRP-2 and c-kit probes showed that strong expression of steel was localised to the targets of migration of TRP-2 expressing cells. One of the most strongly expressing regions in the head was in a band of mesenchyme around the ventral side of the developing cochlea close to where there were high concentrations of TRP-2-positive cells (data not shown). Therefore, the localisation of steel would enable it to be involved in the migration and targeting of TRP-2-expressing cells during normal development.

In the telencephalon, c-kit and steel again appear unlikely to regulate the expression of TRP-2. At 9.5 dpc, c-kit and steel are expressed generally but weakly throughout the prosencephalon, prior to TRP-2 expression. When TRP-2 mRNA is first detectable at a low level in the telencephalon, at 10.5 dpc, c-kit expression diminishes in the same region, whilst remaining weakly expressed in the adjacent part of the telencephalon. Subsequently, at 12.5 dpc, c-kit is localised in a pattern complementary to TRP-2 (Fig. 4C,D,F,G). Flanking the TRP-2 domain, c-kit is expressed across the whole thickness of the neurepithelium. Within the frontal region of the telencephalon, TRP-2 transcripts are confined to the germinal layer, whilst c-kit is expressed in the enveloping marginal layer (Fig 4C,D). Overlaying the whole of the telencephalon we see expression of steel in the mesenchyme, and also strong expression in the floorplate of the neural tube. Therefore, the lack of a spatial correlation between the c-kit and steel mRNAs suggests that any interaction between the two gene products is not likely to be directly responsible for the activation of TRP-2.

Expression of TRP-2 in Steel-dickie *mutant embryos*

We have used the TRP-2 probe to examine the way in which melanocyte development fails in Sl^d/Sl^d embryos. Homozygous mutants were distinguished from their heterozygous and wild-type littermates in segregating litters using Southern analysis and PCR techniques, as described in the Materials and methods.

The telencephalon shows the normal pattern of TRP-2 expression throughout development in Sl^d homozygous embryos (Fig. 5A,B). This supports our assertion that the interaction between c-kit and the membrane-bound form of the steel factor, missing in *Steel-dickie* mutants, does not play a role in activating or maintaining TRP-2 expression. Similarly, TRP-2 expression in the developing endolymphatic duct and in the PRE appeared normal in the mutant embryos (Fig. 5C-F). These findings of hybridisation with TRP-2 in mutant embryos provide a positive control for our observations of melanoblasts in the same sections. Serial sections allowed us to examine the whole head region of each of the embryos.

At 11 dpc, all five mutants that we studied had a considerable number of TRP-2 labelled melanoblasts, at least half as many as seen in stage-matched littermate controls. In fact, the group of control mice varied in their abundance of melanoblasts due to slight differences in their rate of development and the fact that the number of TRP-2-positive cells increases rapidly at around this time of development. Some of the littermate controls were heterozygous for the Sl^d mutation, and this may also have contributed to the variability. The distribution of the melanoblasts in the mutants was broadly similar to that in the controls - they were in the mesenchyme between the eye and neural tube and many were found caudal to the otic vesicle (Fig. 6A,B) - but the clustering of these cells close to the developing inner ear seen in controls by 11 dpc was not generally found in the mutants. The melanoblasts shown in Fig. 6D are the closest to the inner ear that were seen in any of the 11 dpc Sl^d/Sl^d embryos.

One day later, at 12 dpc, one mutant had fewer than 10 melanoblasts detectable in the whole head and these were mostly caudal to the otic vesicle. The second mutant at 12 dpc had more TRP-2-positive cells, with a small cluster in the mesenchyme dorsolateral of the neural tube and anterior to the eye, and another group caudal to the ear around the anterior cardinal vein. Littermate controls all showed an abundance of labelled cells, with many clustered around the inner ear (Fig. 6E,F).

By 13 dpc there were very few TRP-2-labelled cells found, fewer than ten in both of the mutants examined, and in the 14 dpc mutant only two or three poorly labelled cells were seen in the whole head. In postnatal specimens, no melanoblasts were identified (data not shown).

Melanoblasts in Sl^d/Sl^d mutants thus appear to begin to migrate from the neural crest normally and begin to differentiate normally, insofar as their transcription of the TRP-2 gene indicates, but from around 11 dpc they start either to die or to dedifferentiate. The cell surface form of the steel gene product, which is missing in *Steel-dickie* mutants, therefore appears to be important for the survival of melanoblasts. In addition, the melanoblasts found caudal of the otic vesicle generally fail to migrate rostrally towards the vesicle as occurs in normal mouse embryos at the same age, suggesting that membrane-bound steel factor is involved in this stage of targeting of migrating melanoblasts. The latter suggestion is supported by our finding of steel transcripts in a band of mesenchyme immediately adjacent to the ventral part of the normal otic vesicle at about the same time (data not shown).

Discussion

We have shown that TRP-2/DT can be used as an early marker for melanocytes, detecting migrating melanoblasts as early as 10 dpc, which is 4 days before tyrosinase and TRP-1 probes detect melanocytes and 6 days before they can be identified by the production of visible pigment. TRP-2/DT should thus prove to be a useful tool for analysing melanoblast migration in normal and abnormal conditions. The availability of neural crest markers in birds, such as chick/quail transplants, the HNK-1 antibody and the GlN1 monoclonal antibody, has been extremely useful in elucidating the pattern of neural crest cell migration and the factors influencing migration and differentiation (e.g. Stern et al., 1991; Barbu et al., 1986). In mammals, the detailed pattern of neural crest migration is likely to differ from that of birds; for example, the bird inner ear is quite different from that of mammals and no neural-crest-derived melanocytes have been described in the bird equivalent of the stria, the tegmentum vasculosum. Neural crest cell markers previously described in mammals include the trancription factor AP-2, which detects a wide range of neural crest derivatives, the B30 ganglioside, which is a marker for neural-crest-derived neurons, the trk proto-oncogene, which is expressed only in sensory cranial and dorsal root ganglia of neural crest origin, and the MASH-1 clone, which is expressed in peripheral neuronal cells (Mitchell et al., 1991; Stainier et al., 1991; Martin-Zanca et al., 1990; Johnson et al., 1990). c-kit expression has been described in individual cutaneous cells starting from between 12.5 and 14.5 dpc (Keshet et al., 1991; Orr-Urtreger et al., 1990; Nishikawa et al., 1991). Manova and Bachvarova (1991) reported c-kit-labelled cells in the skin from 10.5 dpc, but these early cells may represent other c-kit-positive cells such as mast cell precursors; transplantation experiments show that some areas of the skin have very few if any melanoblasts at 11 dpc (Rawles, 1947; Chui et al., 1976). In our own hybridisations with c-kit, the earliest that individual melanoblasts were clearly detected was at 13.5 dpc, and some areas of mesenchyme that were not spatially related to melanocytes were also labelled with c-kit, suggesting that at this age the labelling was not confined to melanoblasts. The histochemical dopa-premelanin reaction is reported to reveal melanoblasts only from 14.5 dpc (Hirobe, 1984). Thus, TRP-2/DT is the first mammalian neural crest cell marker that clearly identifies the melanocyte cell lineage from a very early stage.

We found widespread distribution of steel and c-kit transcripts in the head region of normal embryos, with localisations broadly similar to those reported previously by other authors (Orr-Urtreger et al., 1990; Matsui et al., 1990; Manova and Bachvarova, 1991; Keshet et al., 1991; Motro et al., 1991). Our main interest in examining steel and c-kit localisations was to make a detailed comparison with TRP-2 distribution in adjacent sections, and this led us to three main observations. Firstly, the pattern and timing of steel and c-kit expression suggested that any steel-c-kit interaction is not responsible for the early differentiation of melanoblasts or for the onset of TRP-2 expression in the telencephalon. The comparison between c-kit-labelled cells and TRP-2-labelled cells suggested that c-kit-labelled cells were probably not all melanoblasts. Finally, the localisation of steel expression, particularly in the mesenchyme close to the developing cochlear duct where there were many TRP-2-labelled cells, suggests that steel may be involved in the migration and targeting of melanoblasts.

We do not know what function the TRP-2 product, DT, might have in the developing telencephalon, if indeed the mRNA is translated into a functional enzyme there. One speculation is as follows. The unrelated tyrosine-metabolising enzyme tyrosine hydroxylase (TH) begins to be expressed around the time that we see TRP-2/DT expression, at 12 dpc in the rat which is equivalent to around 11 dpc in the mouse (Coulon et al., 1990). The product of TH is DOPA, which is rapidly converted to dopamine by the action of dopa decarboxylase (DDC). It is possible that activation of DDC occurs later than TH, which would normally lead to the accumulation of potentially toxic melanin precursors spontaneously from DOPA. DT might then act as a detoxification mechanism if it is turned on in the same cells as TH, where it may act on any DOPAchrome made to convert it to a less toxic intermediate (Tsukamoto et al., 1992). It is also not clear why TRP-2 is expressed in the endolymphatic duct, although presumably the enzyme could have a detoxification role here too.

The earliest expression of TRP-2 was found at 9.5 dpc in the optic vesicle in the region that we presume was destined to become the PRE. TRP-1 expression was also detected earlier here than elsewhere in the embryo, at 11.5 dpc, and tyrosinase transcripts were seen from 13.5 dpc. As pigment could be seen at 11.5 dpc in the PRE, we believe that tyrosinase enzyme must have been produced earlier and that our failure to detect its mRNA was probably due to low levels of the message being present. The onset of TRP-2 expression corresponds well with the timing of pigment cell commitment in the developing optic vesicle described by Buse and de Groot (1991). They found that eye anlages taken from 9.5 dpc embryos or earlier cultured in isolation remained almost free of pigment, while anlages from 10 dpc onwards developed pigment epithelium. This suggests that TRP-2 expression occurs at the same time as, or even slightly earlier than, optic vesicle cells become committed to become pigment cells.

The transcriptional regulation of TRP-2/DT is clearly dif-

ferent from that of tyrosinase and TRP-1. It will be interesting to determine whether the much earlier activation of transcription of TRP-2/DT is due to a qualitative difference in gene regulation, using different or additional transcription factors, or is a quantitative difference such that the TRP-2 gene is more sensitive to the same factors. The reason for the very early activation of TRP-2/DT in melanoblasts is not clear, as there is no evidence that it is a factor required for further differentiation of the cells (Tsukomoto et al., 1992, Jackson et al., 1992). The gene product, DOPAchrome tautomerase, may be required for detoxification in melanoblasts in the same way as we speculated for the telencephalon and thus perhaps must be made in advance of tyrosinase.

We found relatively few melanoblasts in the developing skin until around the third week of gestation, and there were large areas of skin in younger embryos in which none could be identified. Our findings are primarily restricted to the head region, but they may nonetheless be relevant to the mechanism of coat colour development in chimaeras. Chimaeric animals made by mixing cells from embryos of different coat colour genotype or by retroviral marking of albino melanoblasts to restore tyrosinase activity do not have a finely grained mixture of hair colours, but rather have large transverse patches of hair of the same colour which usually do not cross the dorsal midline. This indicates that large areas of the coat are pigmented by descendents of single precursors (Mintz, 1967; McLaren, 1976; Bradl et al., 1991; Huszar et al., 1991). Our observations of very few cutaneous melanoblasts in early development support this suggestion, at least in the head region, and further suggest that much of the melanoblast proliferation occurs after migration.

The *Steel-dickie* mutant lacks the cell surface, membrane-bound form of the steel gene product, and it might be predicted that it is this form, rather than the soluble form, that would be involved in targeting of migratory cells. Our finding that TRP-2-labelled cells did not often appear close to their final target sites in *Steel-dickie* mutant embryos supports this proposition. Our finding that presumptive melanoblasts do not survive for more than a few days in *Steel-dickie* embryos further suggests that the cell surface form of the steel factor is required for melanoblast survival. The depletion of melanoblasts between 11 and 14 dpc in *Steel-dickie* mutants cannot be accounted for simply by a failure of these cells to proliferate, because we examined serial sections through the entire head (except for a small number used for control incubations) and we would have detected the melanoblasts had they survived in a recognisable form. On the other hand, early differentiation and early migration of melanoblasts appeared normal in mutant embryos, which indicates either that the steel factor is not required for these steps or that any soluble form produced by *Steel-dickie* mutants is sufficient.

Mutations at the *Steel* locus also affect other migratory cells, such as primordial germ cells (pgcs) and mast cells. McCoshen and McCallion (1975) reported that pgcs in presumed *Sl/Sl*^{d} mutant embryos appeared to migrate normally at first towards the genital ridges but were fewer in number than in controls due to a failure to proliferate or to cell death. Godin et al. (1991) have used cultured pgcs to show that the steel gene product is essential for their survival, and Dolci et al. (1991) have further shown that it is the membrane-bound form that is the survival factor for cultured pgcs and that the soluble form has only a limited ability to support their survival. Pgcs thus appear to be similar to melanoblasts in requiring the membrane-bound steel gene product for survival. Mast cells, in contrast, have been reported to survive and proliferate in the presence of either the soluble or the membrane-bound form of the steel gene product (Dolci et al., 1991; Anderson et al., 1990). Thus, different cell lineages have different requirements for the two forms of the steel gene product, and an improved understanding of these requirements will help to explain the phenotypic differences observed between different *Steel* mutants.

During the second half of gestation in the mouse, melanoblasts are particularly prominant around the inner ear. The migration of melanocytes to the inner ear is a developmentally important process because in addition to their pigment-producing function, neural-crest-derived melanocytes have an important but enigmatic role in the inner ear. In the cochlea they are mainly localised to the stria vascularis, which in normal mice produces a resting potential of about 100 mV in the lumen. In mice homozygous for some of the mutations of either *W* or *Steel*, no melanocytes can be identified in the stria vascularis, the stria is dysfunctional and no potential is generated, resulting in severe hearing impairment (Deol, 1970; Steel and Barkway, 1989; Steel et al., 1987; Schrott et al., 1990). The function of melanocytes in the cochlea is additional to their production of pigment, as albino animals, which have normal but unpigmented melanocytes in their stria, have normal cochlear function. In *Steel-dickie* homozygotes, no melanocytes can be identified in the postnatal stria vascularis but there are degenerating cells present for the first few days after birth (Grainger, Barkway and Steel, unpublished data). We wondered if these degenerating cells represented dying melanocytes, but the present study suggests that melanoblasts never reach the stria and disappear long before birth. Melanocytes in the inner ear are of clinical importance because there are a number of forms of deafness with associated spotting-type pigmentation defects such as piebaldism and Waardenburg syndrome. It will be interesting to discover if any forms of deafness in humans result from mutations in the recently described human homologue of steel, SCF (Geissler et al., 1991).

We thank Liz Graham, Angela Pearce, Chris Barkway and Jo Cable for their assistance with parts of the processing, Mia Buehr for discussions about germ cells, P. Besmer, G. Schutz, and P. Koopman for the probes, Cami Brannan for advice on PCR of the *Steel* gene and Jo Cable for comments on the manuscript. I.J.J. was a Lister Fellow.

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(Accepted 20 April 1992)